86-Plat
Characterization of Cholesterol and Drug Ligand Interactions with Translocator Protein 18 KDA (TSPO) from *Rhodobacter Sphaeroides*
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The mitochondrial outer membrane protein Translocator Protein 18 kDa (TSPO), previously known as the peripheral benzodiazepine receptor (PBR), has been proposed to be a key component of the cholesterol transport system in mitochondria and a rate limiting step for steroidogenesis. Dysregulation of neurosteroids generation involving TSPO has been shown to be related to a variety of neurological and psychiatric diseases, including Alzheimer's disease, Parkinson's disease, as well as bipolar disorder. In addition, ligands of TSPO are widely used for imaging brain injury and inflammation, areas in which TSPO is highly expressed. A natural human single-nucleotide polymorphism (SNP) gives rise to a mutation, A147T, within the proposed cholesterol recognition site (CRAC). This mutation leads to altered ligand binding and has recently been reported to reduce pregnenolone production and to be associated with bipolar disorder. The same mutation is reported to inhibit a proposed TSPO catalyzed degradation of prototoporphyrin IX. TSPO from *Rhodobacter sphaeroides (Rs*TSPO) shares high sequence similarity with the human protein but differs in the region of the SNP and shows significantly lower cholesterol binding. To better understand the interaction of TSPO with cholesterol, porphyrin and other ligands, we expressed and purified the recombinant *Rs*TSPO and several mutants in the cholesterol binding region. Binding properties were investigated with a sensitive tryptophan fluorescence quenching assay and the interactions were characterized by X-ray crystallography.(Supported by the MSU Center for Mitochondrial Science and Medicine, a Strategic Partnership Grant from Michigan State University Foundation and GM26916 to SFM).

87-Plat
Monitoring Intramembrane Proteolytic Cleavage Reactions using Isotope-Assisted Vibrational Interrogation of Membrane Embedded (IVIBE) Proteins
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While several intramembrane cleaving proteases (iCliPs), enzymes that carry out proteolysis reactions within the membrane interior, have had their structures solved, basic biochemical questions such as what determines a substrate and how cleavage site is dictated remain. However, our inability to monitor enzyme-substrate interactions in a lipid environment is the dominant factor limiting our understanding of the central questions associated with these enzymes. Specifically, while there are many excellent techniques for the characterization of membrane proteins, many of them are only amenable to steady state measurements, require removal from the membrane, or are too low resolution to offer detailed information about changes in structure and environment. Here we offer a new method by which to observe structural fluctuations of the enzyme and the substrate during cleavage reactions within a membrane environment: isotope-assisted vibrational interrogation of bilayer-embedded systems (iVIBE). Deep UV resonance Raman (DUVRR) spectroscopy has previously been used to observe structural and environmental changes in both soluble and membrane proteins. Now, using an isotopically labeled protease and an unlabeled substrate we can resolve the spectral responses from each protein, allowing us to observe the cleavage reaction over time and determine the binding site, or structural fate of the substrate.

88-Plat
Investigating Ligand-Modulation of GPCR Activation Pathways
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Molecular dynamics simulations can provide tremendous insight into atomistic details of biological mechanisms, but micro- to milliseconds timescales are historically only accessible on dedicated supercomputers. We demonstrate that cloud computing is a viable alternative, bringing long timescale processes within reach of a broader community. We used Google's Exacycle cloud computing platform to simulate an unprecedented 2 milliseconds of dynamics of the β2 adrenergic receptor (β2AR)_a major drug target G protein-coupled receptor (GPCR). Markov state models aggregating these independent simulations into a single statistical model are validated by previous computational and experimental results and provide the first atomistic description of multiple GPCR activation pathways. We show that agonists and inverse agonists interact differentially with these pathways, creating an opportunity for developing drugs that interact more closely with diverse receptor states, for overall increased efficacy and specificity.

89-Plat
Crystal Structure of MraY, an Essential Membrane Enzyme for Bacterial Cell Wall Synthesis
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The Phospho-MurNAc-pentapeptide translocase (MraY) is a prokaryotic membrane-spanning enzyme involved in an essential process of peptidoglycan synthesis: transfer the precursor phosphor-MurNAc-pentapeptide to carrier lipid undecaprenyl phosphate. MraY belongs to a subfamily of the polyprenyl-phosphate N-acetyl hexosamine 1-phosphate transferase (PNPT) superfamily whose members are involved in various biological processes including eukaryotic N-linked glycosylation. MraY has been a target for antibiotics development for its essentiality and specificity in bacteria. Therefore atomic structure of MraY can provides valuable mechanistic information that can aid development of new antibiotics. We report the crystal structure of MraY from *Aquifex aeolicus* ($MraY_{AA}$), the first structure of the PNPT superfamily, at 3.3 Å resolution. The crystal structure, together with crystallographic and functional studies, reveals the architecture of $MraY_{AA}$, the location of Mg^{2+} at the active site and the putative binding sites of both substrates. Our crystallographic studies provide insights into the mechanism of how MraY attaches a building block of peptidoglycan to a carrier lipid.

90-Plat
Combining Modelling and Site-Directed Mutagenesis to Explore Agonist Binding to Human Orexin Receptors
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Orexin-1 (OX1) and orexin-2 (OX2) are class A G-protein Coupled Receptors (GPCR)s located predominantly in the brain and are linked to a range of different physiological functions, including the control of energy metabolism and regulation of the sleep-wake cycle. The natural agonists for both receptors are two small peptides, Orexin A and Orexin B. Both peptides have activity at both receptors. Although no experimentally-derived structure has yet been published for the receptors in complex with either agonists or antagonists, a large amount of site-directed mutagenesis (SDM) has been reported and has provided important insight into the key determinants of agonist and antagonist activity. In drug-discovery, a working three-dimensional model can provide an intuitive way forward to explore new compounds. Thus we developed homology models that utilized existing SDM data, which we then explored further with MD simulation and ensemble-flexible docking to generate binding poses of the Orexin peptides in the OX receptors. We were then able to test the resulting poses with additional SDM experiments. As part of our modelling procedure we also developed a new method to analyze the structural data generated within an MD simulation to help distinguish between different GPCR substates. Our work demonstrates how this new method of structural assessment for GPCRs can be used to provide a working model for peptide-Orexin receptor interaction.

91-Plat
Kinetic Exclusion Analysis (KinExa) of Avidity Enhancement of a Multi-Valent Adnectin Binding to Clustered Receptors on CHO Cells
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Multivalency is a strategy used in nature to gain avidity. A variety of cell surface receptors are known to cluster at the cell surface via protein or lipid (raft) interactions. Analytical methods to measure the effect of avidity as it exists at a cell surface are challenging. Kinetic Exclusion Analysis (KinExA) is a sensitive immunodetection analytical technique for measuring solution affinity. Adnectins™ are a proprietary type of targeted biologic derived from human fibronectin. Adnectin-A was selected with mRNA display (PROfusion™) to bind specifically to cell surface receptor X, and was formated as a multivalent fusion protein. To determine the affinity and avidity of Adnectin-A for receptor X clustered on cells, both the human and cynomolgus monkey homoloques of

receptor X were transfected into CHO cells. The CHO transfectants were characterized by FACS and then scaled up for KinExA binding studies. KinExA has been used to measure binding affinity of Adnectin-A to the cell surface expressed receptor X to measure the effect of avidity of the multivalent adnectin binding to receptor clusters. As controls for the functional activity of the Adnectin-A and the affinity of the monovalent interaction, the same KinExa assay was used, substituting the soluble receptor X extracellular domain for the transfected CHO cells. The binding avidity measured by KinExA for CHO expressed receptor is 14 pM for both species of receptor X. However, the affinity of Adnectin-A for monovalent soluble Receptor X was quite different between the species suggesting that avidity due to receptor clustering equilizes the functional avidity at the cell surface.

Platform: Voltage-gated K Channels: Activation/ Inactivation Mechanisms

92-Plat

A-Type Kv4 Channel Closed-State Inactivation is Modulated by the Tetramerization Domain Interacting with Auxilary KChIP4a

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A-type Kv4 potassium channels undergo a conformational change towards a non-conductive state at negative membrane potentials, a dynamic process known as closed-state inactivation (CSI). CSI causes inhibition of channel activity without prerequisite of channel opening, thus providing a dynamic regulation of neuronal excitability, dendritic signal integration and synaptic plasticity. However, the structural determinants underlying Kv4 CSI remain largely unknown. We have recently demonstrated that auxiliary KChIP4a subunit contains an N-terminal Kv4 inhibitory domain (KID) that directly interacts with Kv4.3 channels to enhance CSI. In this study, we utilized the FRET two-hybrid mapping and BiFC-based screening combined with electrophysiology, and identified the intracellular tetramerization (T1) domain that functions to suppress CSI and serves as a receptor for the binding of KID. Disrupting Kv4.3 T1-T1 interaction by mutating C110A within the C3H1 motif of T1 domain facilitated CSI, and ablated the KID-mediated enhancement of CSI. Furthermore, replacing the characteristic C3H1 motif of Kv4.3 T1 domain with the T1 domain from Kv1.4 without the C3H1 motif or Kv2.1 with the C3H1 motif resulted in channels functioning with enhanced or suppressed CSI, respectively. Taken together, our findings reveal a novel role of the T1 domain in suppressing Kv4 CSI with the C3H1 motif functioning to stabilize the channel activation gate; and KChIP4a KID directly interacts with the T1 domain to relieve the stabilization, leading to facilitation of CSI and inhibition of channel function.

93-Plat

Two-in-One: Activation and Inactivation at the Intracellular Gate of a Kv Channel

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N-type and P/C-type inactivation are firmly established mechanisms of inactivation in voltage-gated K^+ (Kv channels). However, Kv4.x channel complexes, which undergo fast preferential closed-state inactivation (CSI; Fineberg et al., 2012, *JGP* 140.5:513-527), appear to use a distinct but unknown inactivation mechanism. Previously, we hypothesized that a weak interaction between the voltage sensing domain and the intracellular activation gate underlies CSI (Bähring & Covarrubias, 2011, J Physiol 589:461-79). Thus, CSI is essentially governed by the intracellular activation gate, which fails to open and adopts an inactivated conformation. To directly test this hypothesis, we investigated the heterologously expressed Kv4.1 ternary channel complex including accessory subunits KChIP1 and DPP6, and exploited the ''trap-door'' paradigm of the activation gate. The results show that Kv4.1 inactivation traps intracellularly applied quaternary ammonium blockers (bTBuA and TBuA) inside the channel's pore. The trapped blockers can only escape if the channels are opened again by subsequent depolarizations. By contrast, inactivation cannot trap TEA, whose binding kinetics is faster than that of channel gating. Moreover, under identical conditions, a Shaker Kv channel (ShB-T449K) known to exhibit fast P/C-type inactivation cannot trap bTBuA. These findings conclusively suggest that the intracellular activation gate of the Kv4.1 ternary channel complex plays a novel dual role, controlling both activation and inactivation. Supported in part by NIH grant R01 NS032337 (MC).

94-Plat

Development and Validation Studies of Universal Pharmacophore Models for hERG Channel Openers

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The intra-cavitary drug blockade of hERG channel, a common off-target for many drugs, have been extensively studied both experimentally and theoretically. Structurally diverse ligands inadvertent blockade of rapid component of delayed rectifying K^+ currents are potentially pro-arrhythmic and may lead to drug-induced long QT syndrome-LQTS. There are a number of natural strategies for rational drug design; one dubbed the ''passive'' approach avoids block of hERG1 whereas the ''proactive'' strategy designs treatments to activate the channel. While ''passive'' approach has been developed for decades, studies of structural mechanisms of hERG channel activation by small molecules are truly novel. Accordingly, design of the hERG openers or current activators may offer a momentum for modern anti-arrhythmia drug development. Significant number of small molecules with capacity for hERG activation was identified in mandatory hERG screens. To establish possible correlation between activators structure and reactivity, we attempted to construct a universal pharmacophore model for hERG channel openers using PHASE protocol. The biochemical data on 38 K^+ channel activators are used in training and test sets. These compounds span a wide range of structurally different chemotypes with ~10^5-fold variances in binding affinity, which is sufficient for statistically sound model. A developed five sites AAHHR (A, hydrogen-bond accepting, H, hydrophobic, R, aromatic) pharmacophore model has showed reasonable high statistical results compared to other constructed models and was selected for steric and electrostatic contour maps analysis. The predictive power of the model was also tested with 6 external test-set (as true unknowns) compounds. Pharmacophore model is also combined with previously developed receptor-based homology model of hERG K channel and novel activators are generated and screened. The developed ligand-based models may serve as a basis for the synthesis of novel potential therapeutic hERG activators.

95-Plat

N-Terminal Regulation of hERG1 K^+ Channel Deactivation

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Slow deactivation of hERG1 (Kv 11.1) potassium channels maintains I_{Kr} during final repolarization of the cardiac action potential and opposes asynchronous early depolarization. Inherited point mutations in hERG1 that accelerate deactivation of I_{Kr} cause long QT syndrome (LQTS), a disorder of ventricular repolarization that increases the risk of lethal cardiac arrhythmia.

The intracellular N-terminal domain of hERG1 is known to be essential for slow deactivation. Deletion of the entire (~350 residues) or just the initial 16 residues of the N-terminus accelerates deactivation 10-fold. The same effect is achieved by neutralization of the charged residues, Arg4 or Arg5. How many of the 4 N-termini are required to slow channel deactivation is unknown. hERG1, like other Kv channels, is a homotetramer. By repeatedly linking the C-terminal of one subunit to the N-terminal of the next subunit we constructed concatenated hERG1 tetramers. A variety of homomeric and heteromeric concatenated tetramers were characterized (i.e., WTn/R4A:R5A(4-n); where n = 1 to 4). The concatenated channel containing a single R4A/R5A subunit and 3 wild-type subunits deactivated as fast as the concatenated channel containing only R4A/R5A subunits. The LQTS-associated mutation R56Q, located in the N-terminal of hERG1 was also studied. Again, a concatenated tetramer containing a single mutant subunit deactivated as fast as channels with R56Q mutations in all four subunits. Our results show that all 4 N-termini are required to mediate slow deactivation in wild-type hERG1 channels.

96-Plat

Lipid Affinity to the Voltage-Gated Potassium Channel KvAP

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Voltage-gated potassium channels (KV) are formed by a central conducting pore surrounded by four voltage sensor domains. Functional studies have revealed that biophysical properties of lipid molecules in the channels environment can have strong effects on the activity of KV channels. Here, we investigated the influence of different lipids as well as their affinity to KvAP channels. We carried out electrophysiology measurements by fusing vesicles containing purified channels into planar lipid bilayers with varied lipid compositions. We found that KvAP properties are mainly determined by the lipid